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THE EQUILIBRIUM AND STRUCTURE OF LECITHIN-CHOLATE MIXED MICELLES

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The nature of the equilibrium: mixed micelles-intermicellar solution, has been determined for the system Lecithin-Sodium Cholate. The composition of the intermicellar solution has been estimated after light scattering data, and the results confirmed by other methods. The composition and size of the mixed micelles have then been evaluated. Provided that the mixed micelles are in equilibrium with a solution containing at least the CMC of cholate, their size was shown to diminish progressively as the ratio Lecithin/Cholate present in the mixed micelles decreased. At the same time the concentration of the free cholate dissolved in the intermicellar solution, in equilibrium with the mixed micelles, was shown to increase continuously as the size of the mixed micelles decreased. The thermodynamic equilibrium of these systems has been treated in a simple manner. The existence of stable mixed micelles in equilibrium with a free cholate concentration below the CMC was seen to correspond to a metastable state, and in the presence of small amounts of salt such systems immediately coagulated. Finally it is shown that the proposed model satisfactorily accounted for the variation in size of the mixed micelles with the micellar Lecithin/Cholate ratio.

Introduction

Little experimental work has been reported in the literature concerning the composition of mixed micellar systems, and no systematic study has been carried out to establish the equilibrium and the distribution of the components between the mixed-micelles and the solvent. It is known that in such systems the composition of the monomeric solution in equilibrium with the mixed micelles differs from the overall composition of the system, and further that it varies continuously as a particular solution is diluted¹). In order to obtain indirectly the concentration of the monomer in a mixed micellar solution, Mysels and Otter have proposed an empirical interpretation of the conductivity data of two surface active solutes. This enables the composition in the mixed micelles to be evaluated, but not their size¹).

The case of the mixed micelles Lecithin-Cholate appeared a convenient material for the study of such equilibria, as one of the components, Lecithin, is insoluble alone, thus facilitating the determination of the composition in the intermicellar liquid. Using the same assumption as Mysels, that provided the composition of the intermicellar solution does not vary then the composition of the mixed micelles remains unchanged as their number is varied, the free cho-

late composition was determined using indirectly the data of light scattering measurements. This allowed one to deduce both the composition and the size of the mixed micelles. It is probable that the thermodynamic equilibrium described for the present system is similar to that of all systems involving polar solubilisation, but the considerable solubilising capacity of the cholate, and the structural features of the two molecules used, render the present system particularly amenable to a detailed interpretation. The form of the lecithin and cholate molecules and their solubility characteristics are well known. Lecithin is insoluble in water but swells to give a lamellar phase, in which the lecithin is present in repeating bimolecular leaflets (*e.g.* ref. 2). The cholate molecule has a compact steroid-ring structure and a short side-chain with a terminal carboxyl group. The three hydroxyl groups are distributed on one side

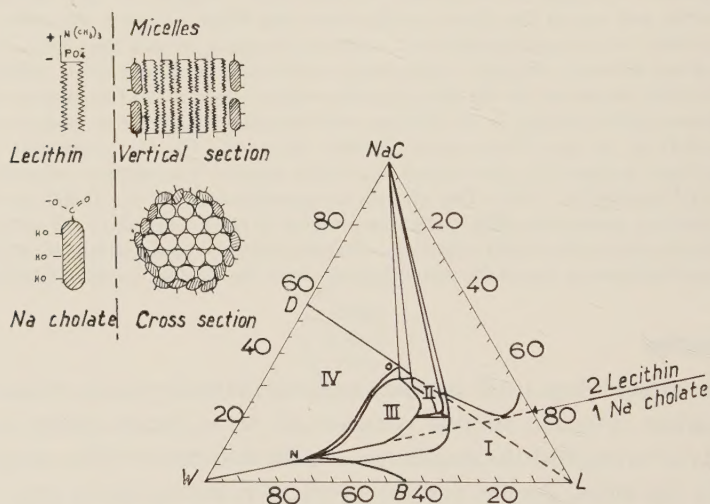


Fig. 1. Lecithin-Sodium Cholate-water ternary phase diagram. I, II, III and IV are the regions of the Lamellar, Cubic, Hexagonal and Micellar phases. The detail shows the proposed model for the Lecithin-Cholate mixed micelles (from the work of Dervichian⁵).

of the steroid structure giving to this face a hydrophilic nature, the opposite face being hydrophobic (*e.g.* ref. 3). The ternary-phase diagram for the components Lecithin-Sodium Cholate-Water has been established recently, fig. 1,⁴ and a tentative model proposed by one of the authors for the mixed micelles (inset fig. 1)⁵. This takes the form of a bimolecular disc of lecithin, each layer of which is stabilised by a ring of cholate molecules covering the external hydrocarbon chains and presenting their hydrophilic face to the water.

The present work was carried out in relation to an enzymatic study of the attack of a lecithinase on lecithin, dispersed with bile salt in the form of

mixed micelles, in which it was shown that the rate of hydrolysis was strongly dependant on the degree of dispersion of the substrate⁶). The present study may also contribute to an understanding of the action of bile salt in the extraction of membrane structures. Finally, the notions of the thermodynamic equilibrium deduced for the present system have been applied to the case of the solubilisation of fatty acids by sodium cholate, which is the subject of a second communication⁷).

Materials and methods

MATERIALS

The preparation and the purification of the egg-lecithin and the sodium cholate have been previously described and the fatty acid composition of the lecithin has also been reported⁴). Other samples of sodium cholate and sodium deoxycholate were obtained from Maybridge Chemical Company, Tintagel, Cornwall, and were chromatographically very pure. There was no difference in the results obtained with the different samples of cholate. The lecithin was stored at 4° in the dark as an alcoholic solution.

PREPARATION OF MIXTURES

The sodium cholate and lecithin were intimately mixed together in the desired proportions in a mutual solvent, *e.g.* methanol or ethanol, and the mixture evaporated to dryness. Water, of *pH* about 10,0 and stored under nitrogen, was then added to give the desired final concentration. Provided that the minimum molecular proportion of cholate needed to solubilise the lecithin was present (1 molecule of cholate for 2 molecules of lecithin) clear solutions were obtained almost instantaneously with this technique. The mode of formation of micellar solutions starting with dry mixtures becomes clear with reference to the three phase diagram established for lecithin-cholate-water systems (fig. 1). The frontier representing the separation between the lamellar and micellar phases occurs at almost exactly 1 cholate:2 lecithins. This is therefore the minimum proportion of cholate necessary to solubilise completely the lecithin in an isotropic phase. The preparation of a solution in the isotropic phase by addition of water to a dry lecithin-cholate mixture, corresponds on the ternary diagram to the displacement of a point on the side NaC-L having the precise L:C composition of the dry mixture, along a line joining this point to the W apex. Unless specifically mentioned in the text, all solutions were prepared in this way.

LIGHT SCATTERING MEASUREMENTS

An apparatus of the type Wippler and Scheibling, modified in the laboratory,

has been used. The intensity of the scattered light was read at angles of 30° , 90° and 150° . In the case of all solutions in the presence of NaCl, the symmetry in the scattering by the solutions was satisfactory. Only in the case of such solutions were detailed calculations on the micellar composition carried out. In cases where only a straight-forward comparison was made between different solutions, the reading at 90° is reported with respect to an arbitrary zero, that of a glass standard in benzene.

The solutions, prepared from dry mixtures in the manner described above, by the addition of distilled water at pH 10.0 to avoid the formation of cholic acid, were rendered dust free by filtration through a $0.45\ \mu$ Millipore filter under nitrogen pressure into cylindrical light scattering cells in 25 ml samples. After each dilution or addition of a product to an initial solution, the mixture was refiltered into a clean cell.

Results

I. VARIATION OF MICELLAR SIZE

The most striking feature of lecithin–cholate micellar systems is the variation in size of the particles as the proportion of lecithin to cholate varies. This is illustrated in the following experiments.

(a) A series of lecithin–cholate solutions containing a fixed quantity of lecithin and varying proportions of cholate were prepared by the addition of a fixed volume of an NaCl solution to dry preparations of lecithin–cholate, previously obtained by mixing the two components together in alcohol and evaporating the alcohol. The dry mixtures dissolved rapidly and the solutions gave steady light scattering readings. Cholate alone scatters little light as its micelles are very small (4–5 m μ). Thus, for a constant lecithin concentration, the variation in light scattering with the different proportions of cholate present gives an indication of the variation in particle size. This variation is shown in fig. 2A, for a fixed lecithin concentration. The light scattering value decreases as the proportion of cholate in the solution is higher. Although the curve is continuous we see that the slope increases very rapidly as the amount of cholate present approaches a minimum value, below which clear solutions could not be obtained. The addition of cholate crystals to any of these solutions brought about a rapid reduction in the light scattering value to a new stable value. The new reading corresponded well to the point on the curve having the same total cholate concentration, *i.e.* in each case for a fixed lecithin–cholate and NaCl composition the same equilibrium light scattering reading was obtained. The solutions were rendered dust-free by filtration after each modification of the composition. Even starting from a preparation of dry lecithin alone, the addition of a cholate–NaCl solution gave an equi-

librium light scattering reading equal to that of the corresponding final composition on the curve.

(b.) We have examined the effect of increasing the electrolyte concentration of lecithin-cholate solutions already containing a certain amount of NaCl.

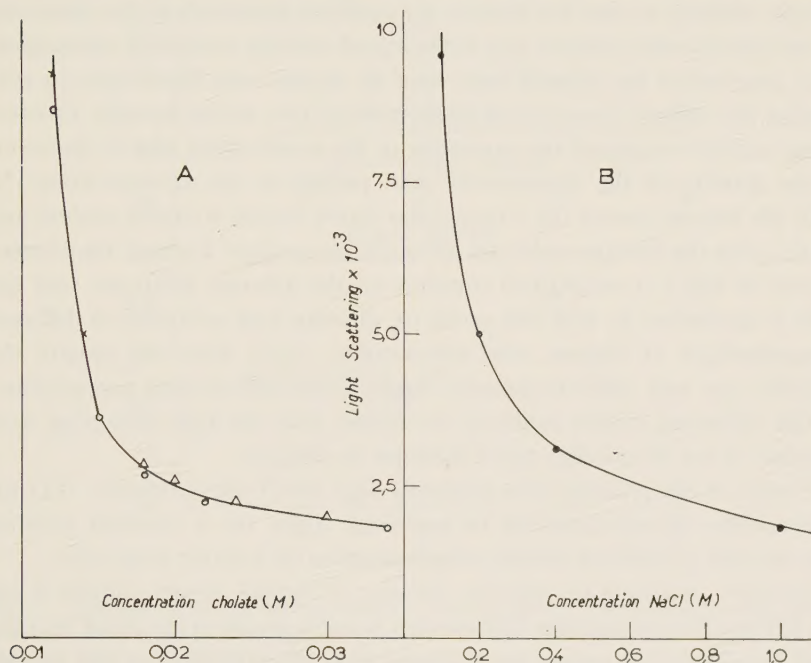


Fig. 2. Variation of light scattering of Lecithin-Cholate solutions. (Lecithin constant 0.01 M.) A: - With increasing Cholate concentration, (NaCl constant 0.12 M). Like symbols correspond to a series of readings obtained by addition of Na Cholate crystals to given initial solutions of Lecithin plus Cholate. B: - With increasing NaCl concentration (Na Cholate constant 0.012 M). The light scattering values reported are with respect to the scattering of a glass standard in benzene.

After each addition of NaCl in the form of solid, the light scattering reading of the solution diminished to a new stable value (fig. 2B). It will be shown later that the effect of NaCl is to decrease the CMC, that is the minimum free cholate concentration necessary for the formation of cholate micelles. This allows equilibrium to be maintained with a lower free cholate concentration, the excess being engaged in the mixed micelles, thus decreasing their size.

II. DETERMINATION OF FREE CHOLATE CONCENTRATION

We have determined the variation in the equilibrium cholate concentration for different particle sizes, by examining the variation in the light scattering

value on diluting the solutions. It is known in the case of mixed micelles that the micellar composition varies upon dilution by water¹). We have made the assumption that if the diluent used has a composition identical to that of the intermicellar solution, then the composition of the mixed micelles would remain constant as they are diluted, the chemical potentials of the molecules in the intermicellar solution and in the mixed micelles remaining unchanged. This assumption has already been used by Mysels and Shedlovsky in estimating the solvent composition in the case of two mixed micellar systems. These authors measured the variations in the conductivity and in the counterion activity of the components with respect to the concentration^{1,8}).

In the present system the intermicellar liquid simply contains cholate and NaCl, since the lecithin molecules are insoluble in water. Further, the concentration of NaCl is maintained constant for the different dilutions. One has then to determine by trial and error, on diluting with solutions of different concentrations of cholate, that composition which does not modify the micellar size, and which is therefore equal to the intermicellar composition.

The following criteria enable us to deduce from the light scattering data whether or not the micellar size is modified by dilution.

Firstly, in the presence of a relatively high NaCl concentration, 0.12 M, intermicellar interactions may be neglected. Thus, for a constant micellar size the light scattered is directly proportional to the number of micelles.

Secondly, although the absolute amount of cholate in each micelle is not yet known, it is enough that this amount remains constant (*i.e.* fixed micellar size) to allow us to record the scattered light as a function of the lecithin concentration, since all the lecithin is engaged in the mixed micelles. Thus, for C_L the lecithin concentration, and S_m the light scattered by the micelles, the ratio C_L/S_m must remain constant when the solution is diluted, if the micellar size does not change. A horizontal line will be obtained by plotting the values of C_L/S_m vs C_L . This will be the case for a diluent having the same composition as the intermicellar liquid. For each concentration the value of S_m is given by the difference $S_m = S_t - S_0$, between the light scattered, S_t , by the solution, and that of the diluent used, S_0 .

If the value of C_L/S_m increases with dilution, this indicates that the size of the micelle decreases, and thus that the concentration of cholate in the diluent is greater than that in the intermicellar liquid. Inversely, if the ratio C_L/S_m decreases with dilution, it means that the cholate concentration of the diluent is lower than that of the intermicellar solution.

For solutions with high lecithin/cholate ratios, the particle size is relatively important, as is the light scattered; and the ratio C_L/S_m is very sensitive to variations of the diluent composition, while for solutions having low lecithin/cholate ratios the ratio C_L/S_m is much less sensitive (fig. 3).

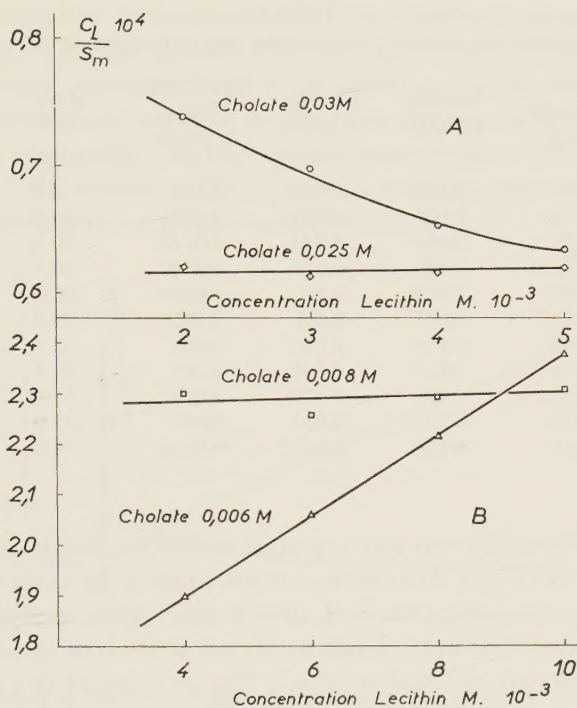


Fig. 3. Dilution curves for determination of the equilibrium free cholate concentration. System A. Lec. 0.005 M, Cholate 0.050 M, NaCl 0.12 M; System B. Lec. 0.01 M, Cholate 0.016 M, NaCl 0.12 M. Ordinate: C_L/S_M , with C_L =concentration of lecithin, and S_M =Light scattering by mixed micelles. Abscissa: gives the lecithin concentration after successive addition of different amounts of diluent. The cholate concentrations of these diluents are marked on the figure, that giving a horizontal plot giving the value of the free cholate concentration in equilibrium with the mixed micelles.

This technique could not be used to determine the free cholate concentration for Lec/Chol systems in the absence of salt. This is explained by the fact that in such systems the intermicellar interactions will be high and will vary considerably with the concentration of the mixed micelles.

We have examined a series of solutions having a constant lecithin concentration (0.005 M), to see how the free cholate concentration in the intermicellar solution varies with increasing proportion of cholate present. The results of six such solutions are shown in fig. 4 (see also in table 1, sol. 1-6).

We have attempted to confirm the order of magnitude of values of the free cholate concentration in equilibrium with the mixed micelles by an independent technique. Lecithin-Cholate solutions were dialysed against different concentrations of cholate (in the presence of NaCl) to determine the external cholate concentration for which cholate did not flow out of the

TABLE 1
Equilibrium cholate concentration, and mixed micellar Wts

Sol. No.	Total molecular ratio, L/C	Lecithin in moles	Cholate in moles	Equilibrium Cholate in moles	MWt $\times 10^{-3}$	Molecular ratio N_L/N_C in micelles
1	0.10	0.005	0.050	0.025	6.8	0.20
2	0.166	0.005	0.030	0.016	15.8	0.36
3	0.25	0.005	0.020	0.0125	30.2	0.67
4	0.33	0.005	0.015	0.0100	40.5	1.00
5	0.50	0.005	0.010	0.0065	100.0	1.42
6	0.625	0.005	0.008	0.0055	220.0	2.0
7	0.625	0.010	0.016	0.008	62.5	1.25
8	0.83	0.015	0.018	0.007	83.5	1.36
9	1.00	0.025	0.025	0.008	82.4	1.47
10	1.00	0.020	0.020	0.007	122.0	1.54
11	0.83	0.010	0.012	0.0058	170.0	1.62

dialysis bag. This value was taken as equal to the free cholate concentration at the interior of the bag. The cholate concentration of the outer solution was measured by colorimetric analysis⁹), after 90 min dialysis, experiments being performed in duplicate and the analysis of each solution being done in duplicate. The results are reported in table 2. The precision of this technique is about 3%. We have verified that very little phospholipid left the interior of the bag, by analysis for P. (Fiske-Subbarow)¹⁰). These values confirm the order of the results obtained by the light scattering technique, although it is not certain that a true equilibrium had been attained in these dialysis experiments.

TABLE 2
Dialysis of Lecithin-Cholate solutions against Na Cholate.
Variation of the Cholate concentration outside the bag

Solution in bag	Initial concentration of external cholate (moles)	External concentration after 90 mn (% of initial value, average of 4 determinations)
A	0.0025	128 $\pm$ 1.0
A	0.0035	108 $\pm$ 1.5
A	0.0045	96 $\pm$ 1.0
B	0.010	111 $\pm$ 1.0
B	0.015	106.5 $\pm$ 2.0
B	0.020	98 $\pm$ 2.0

A⁽¹⁾ Lec. 0.005 M; Cholate 0.008 M; NaCl 0.10 M.

B⁽²⁾ Lec. 0.005 M; Cholate 0.050 M; NaCl 0.12 M.

(1) Less than 1% P detected outside sack after 48 hr.

(2) Less than 1% P detected outside sack after 1 hr.

Free Cholate concentration: Solution A = 4.0-4.5 mM; Solution B = $\sim$ 20 Mm.

It can be seen that the concentration of free cholate is dependent on the total amount of cholate present. This free concentration is the greater the smaller the light scattering value of the solution. Further, as the limiting cholate concentration found to be necessary for the formation of stable solutions is approached, the free cholate concentration tends towards the value of the CMC of cholate. (This is equal to 5 mM as determined by surface tension measurements in the presence of NaCl 0.12 M.)

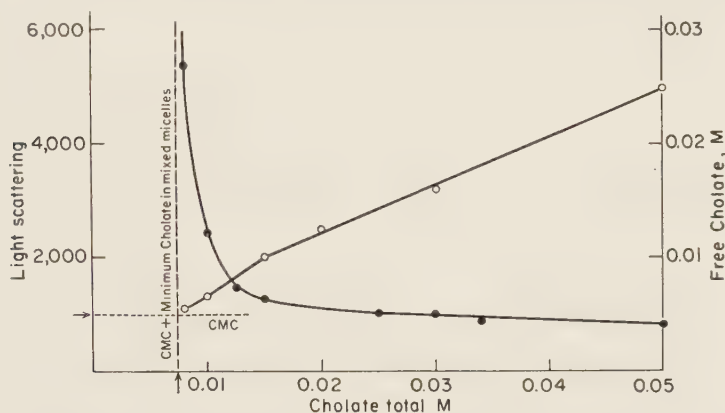


Fig. 4. Variation of light scattering and free cholate concentration with varying total cholate concentration (lecithin constant 0.005 M). ... Light scattering. $\circ\circ\circ$ Free cholate concentration. The vertical line at 0.0075 M cholate is the minimum cholate necessary to provide a 1:2, cholate: lecithin ratio in the mixed micelles (2.5 mM cholate 5.0 mM lecithin) together with a free cholate concentration equal to the CMC (5 mM). The light scattering curve is asymptotic to this vertical line.

We can conclude that in the presence of salt, stable mixed micelles may be formed only when the intermicellar solution contains at least enough cholate to give the CMC. The maximum micellar size will be that in equilibrium with exactly the CMC of cholate, and as the free cholate concentration increases above the CMC the ratio N_L/N_c in the mixed micelles and the size of the mixed micelles, decrease progressively.

The limiting ratio N_L/N_c , corresponding to the maximum micellar size, approaches the value of 2.0 (A clear confirmation of this value is reported later). For a lecithin concentration of 5 mM this gives 2.5 mM cholate in the mixed micelles, in equilibrium with cholate 5 mM in the intermicellar solution. Thus we see in fig. 4 that the light scattering curve is asymptotic at a total cholate concentration of $2.5 + 5.0 = 7.5$ mM.

One can now explain why, as this limiting value of cholate necessary for stable solutions is approached, the slope of the light scattering curve increases abruptly. In fact in this region almost all the cholate present must be

in the intermicellar solution to provide a concentration at least equal to the CMC. Thus, any small decrease in the total amount of cholate in the mixture results principally in a decrease in the proportions of cholate to lecithin in the mixed micelles, and consequently to an increased light scattering. A more exact picture of the variation of the scattered light with particle size is given by plotting the light scattering values of fig. 4 against the Lec/Chol ratio actually in the mixed-micelles, N_L/N_C , obtained by subtracting the free cholate concentration from the total cholate (Fig. 5).

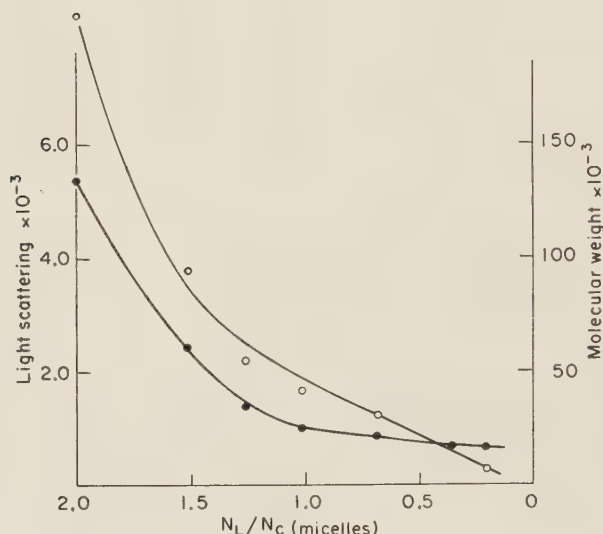


Fig. 5. Light scattering and micellar weight values vs molecular ratio, N_L/N_C , in the mixed micelles. (Lecithin constant 5 mM). ... Light scattering. ooo micellar weights.

A demonstration of the validity of this approach to determine the free cholate concentration is given in the following manner:

In fact, if the free cholate concentration depends only on the size of the mixed micelles and not on their number, knowing the variation of the light scattering as a function of the total concentration of cholate for a given fixed amount of lecithin, it is simple to calculate this variation for other fixed quantities of lecithin.

Let L be the concentration of Lecithin, and C^L the corresponding cholate concentration in the mixed micelles. If x is the concentration of free cholate, its total concentration is $(C^L + x)$.

The light scattering S , due to the solvent and to the micelles, may be written:

$$S_{t(\text{total})}^L = S_{m(\text{micellar})}^L + S_{0(\text{solvent})} \quad (1)$$

Doubling the number of micelles (which necessitates doubling the amount of lecithin), the light scattering becomes

$$S_t^{2L} = 2S_m^L + S_0 \quad (2)$$

since the scattering by the intermicellar liquid remains unchanged. In general if the number of mixed micelles is increased by a factor “ n ”,

$$S_t^{nL} = nS_m^L + S_0 \quad (3)$$

and substituting for S_m^L of eq. (1)

$$S_t^{nL} = nS_t^L - (n-1)S_0. \quad (4)$$

But to conserve a fixed micellar size, the ratio L/C in the micelles must be constant. Thus when the quantity of cholate is nL , the total amount of cholate is ($nC_t^L + x$).

Using the value of S^L and S_0 determined for a series of solutions with Lec 0.005 M (Fig. 4) we have computed the theoretical curves of: light scattering vs cholate concentration for Lec 0.01 M; and 0.015 M ($n=2$, $n=3$). (See fig. 6, table 3.) The light scattering values of a number of such solutions have been measured, and are seen to coincide very well with the predicted values.

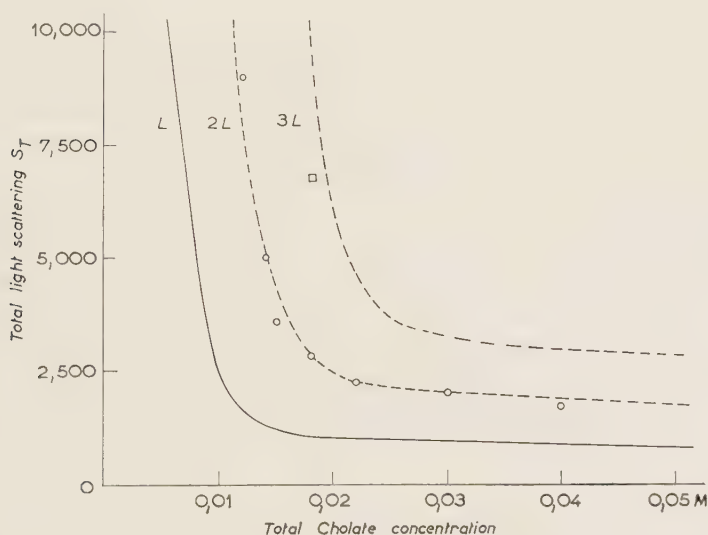


Fig. 6. Theoretical light scattering curves as a function of the cholate concentration, for different lecithin concentrations. — Experimental curve of fig. 4 (Lecithin 5 mM) — — — Calculated curves for lecithin 10 mM and 15 mM (2L and 3L of table 3). ○ and □ Experimental values for solutions of lecithin 10 mM and 15 mM respectively.

TABLE 3
Predicted light scattering values of solutions, at constant micellar
size, increasing number of micelles

Experimental values Lecithin L=0.005 M				Predicted values Lec. 2L=0.01M Lec. 3L=0.015M			
C^L+x	S_t^L	S_0	x	$C^{2L}+x$	S_t^{2L}	$C^{3L}+x$	S_t^{3L}
0.008	5400	49	0.005	0.11	10.750	0.014	16.100
0.010	2440	51	0.0055	0.0145	4.830	0.019	7.220
0.015	1250	62	0.010	0.0200	2.440	0.025	3.630
0.020	1060	70	0.0125	0.0275	2.050	0.035	3.040
0.030	960	95	0.0160	0.044	1.820	0.058	2.690
0.050	800	157	0.0250	0.075	1.440		

C^L+x ; $C^{2L}+x$; $C^{3L}+x$; = Total cholate concentration for Lecithin 5, 10, 15 mM...

S_t^L , S_t^{2L} , S_t^{3L} ... Light scattering of solutions for Lecithin 5, 10 mM...

S_0 = experimental values of light scattering by pure cholate solutions having the concentration of the intermicellar liquid, x , measured for Lec-Chol solution with Lec fixed 5 mM.

One can conclude that whatever the overall concentration of a solution, a certain mixed micellar size, related to the lecithin–cholate ratio N_L/N_C in the mixed micelles, is in equilibrium with a fixed free cholate concentration $\geq$ CMC of cholate.

III. ESTIMATION OF MICELLAR WEIGHTS

Generally in the determination of the Micellar Weights, in order to eliminate the effect of interactions, the scattering of the light is measured at several dilutions, and the value at zero concentration, obtained by extrapolation, is used in the calculation. This procedure could not be used with mixed micelles. In order to determine the micellar weight, measures have been made at one concentration, but in the presence of salt 0.12 M, since under these conditions micellar interactions become negligible. Nevertheless, the dilution technique was used to establish the equilibrium concentration of the intermicellar cholate, as described in the previous section. Thus for each solution, not only the light scattering value S_t at 90° was measured, but also that of a pure cholate solution having the concentration of the inter-micellar liquid, S_0 . The scattering, S_m , to be attributed to the mixed micelles alone can then be obtained by simple subtraction, $S_m = S_t - S_0$. S_m may then be substituted in the equation

$$MW = 5.93 \times 10^{-3} \frac{1}{(dn/dc)^2} \frac{S_m}{C_m} \quad (5)$$

to determine the weight average MWts of the mixed micelles. To justify the use of readings at only 90° it was verified in all cases that scattering of solu-

tions at angles symmetrical with respect to 90° (i.e. 30° and 150°) were almost identical.

C_M , the total weight concentration of the mixed micelles, (g/ml), is obtained by subtracting the cholate concentration of the inter-micellar solvent, from the total weight concentration, (cholate + lecithin) in the initial solution. The constant term has been evaluated for the apparatus using the value of the light scattered by a glass standard. The increment of the index of refraction will be the source of a slight error which escapes our control, as the dn/dc values were determined by refractometer on solutions having a concentration as high as 10% with the same solute ratio as the more dilute solutions used in the light scattering experiments.

The MWts values and free cholate concentrations determined for a number of solutions are reported in table 1 and in fig. 7. It can be seen that irre-

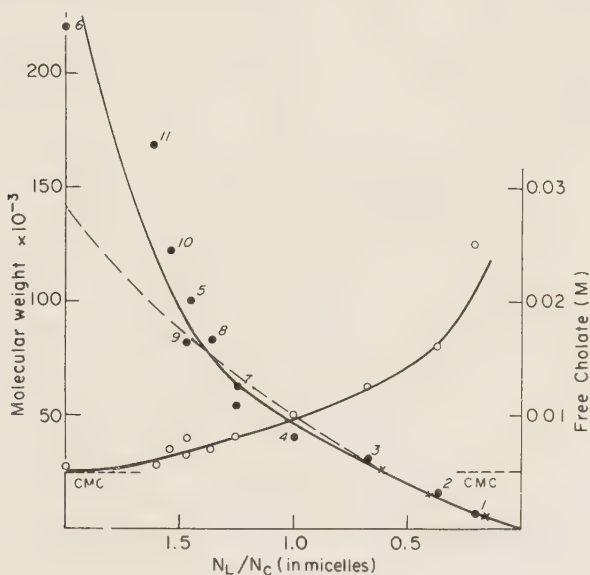


Fig. 7. Micellar weights and free cholate concentrations vs molecular ratio N_L/N_C in mixed micelles. ... Micellar weights. $\circ\circ\circ$ Free cholate concentration. $\times\times\times$ Micellar weights estimated from analytical centrifugation (after Small, ref. 15). Broken line $---$, micellar weight of the proposed model for the same N_L/N_C ratios.

spective of the total lecithin concentration of the solutions, these values fall on smooth curves, and would appear to be uniquely a function of the lecithin-cholate molecular ratio in the mixed micelles (N_L/N_C).

It is seen that the micelles have a maximum size when the free cholate concentration in equilibrium corresponds to the CMC. On the same figure we

see that the ratio N_L/N_C , the number of molecules of lecithin to cholate in the micelles, is then equal to 2. When the ratio N_L/N_C decreases, the micellar size decreases rapidly at first, while the free cholate concentration increases slightly above the CMC. Then for progressively smaller N_L/N_C ratios the size of the micelles falls gradually to around 10000, the concentration of cholate in equilibrium becoming bigger and bigger. A simple thermodynamic explanation of this behaviour will be presented after we have examined the importance of the minimum cholate concentration for the stability of the mixed micelles.

IV. "NON-SATURATED" LECITHIN-CHOLATE SOLUTIONS

In the case of all the solutions considered above, in the presence of NaCl, the free cholate concentration was found to be greater than or equal to the CMC of cholate in 0.12 M NaCl (5 mM). Any dry mixture which did not contain enough cholate in order that, after addition of water, it could assure both a concentration of cholate equal to the CMC in the intermicellar solution, and a ratio of 1 Cholate to 2 Lecithin in the micelles, could not give a clear solution.

On the contrary, in the absence of salt, clear solutions could often be obtained, provided the ratio of Chol/Lec in the dry mixture was above 1:2 even for a cholate concentration lower than the CMC in the intermicellar solution. As can be seen in the ternary diagram 1 chol:2 lec is the minimum ratio necessary to solubilise completely the lecithin as an isotropic phase. In the case of all concentrated solutions the quantity of cholate needed to maintain the CMC in the water is negligible with respect to the total cholate present. Thus, this frontier 2L:1C on the ternary diagram gives the limiting L/C ratio in the mixed micelles themselves. However, as much more water is added to a concentrated system, the cholate needed to provide an intermicellar solution at the CMC will represent an increasingly high proportion of the total cholate present. Eventually there would be insufficient cholate to assure simultaneously the minimum value in the micelles and a solution at the CMC. This can be demonstrated by a simple calculation. For example, starting with a dry mixture having a L/C ratio 1:1, in 70% water less than 3% of the total cholate present is needed to give the CMC in the intermicellar solution. But in 97% water, half of the total cholate must be present in the water to give the CMC, and just enough remains to provide the limiting ratio in the mixed micelles. Above 97% water, therefore, the micelles or the solution, or both, will be deficient in cholate. This is true irrespective of the mechanism by which the mixed micelles may change their composition on diluting a solution. As we have clearly demonstrated that under such conditions clear solutions were not obtained in the presence of salt, it is interesting

to study the properties of these dilute aqueous solutions in the absence of NaCl, by addition of sodium cholate crystals and of salt.

a) Addition of Na cholate crystals

For a fixed lecithin concentration of 0,01 M, the minimum amount of cholate needed to give an overall C/L 1:2 ratio is 0,005 M. The cholate necessary to provide this ratio in the micelles, and assure also a concentration equal to the CMC in the intermicellar solution (0,012 M) is therefore $0,005\text{ M} + 0,012\text{ M} = 0,017\text{ M}$. A number of solutions were prepared corresponding to the points No. 1–5 on the curve (A) (fig. 8), containing cholate concentrations falling between these two limits 0,005 and 0,017 M. It was observed that the addition of cholate crystals to such solutions brought about a decrease in the light scattering value, as would be expected, but only when a certain threshold value of added cholate was exceeded. On the contrary, when cholate was added below this threshold value, an increase in the light scattering

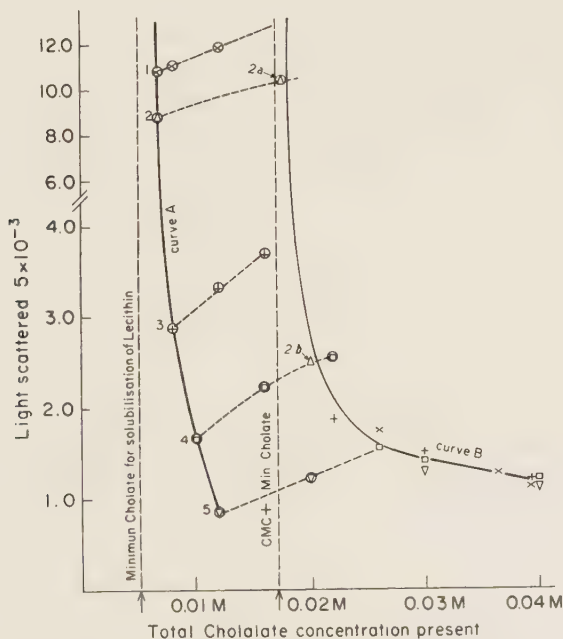


Fig. 8. Effect of addition of Sodium Cholate to dilute aqueous Lecithin-Cholate solutions. Water was added to dry anhydrous mixtures of lecithin plus cholate, giving the solutions numbered 1 to 5, having a constant lecithin concentration 0.01 M, and cholate concentrations lying between the limits 5 mM and 17 mM. Amounts of sodium cholate crystals were added successively to each solution, and the light scattering values followed as the total cholate concentration increased. (See text for description of this behaviour.) (Solutions 2, 2a, 2b were prepared respectively by addition of water (2), cholate 11 mM (2a), cholate 13 mM (2b), to identical dry lecithin cholate mixtures).

value was first observed, and the new reading was stable over 24 h (table 3a, b, c). When the total amount of added cholate corresponded to an increase in the cholate concentration of the order 0.013–0.014 M, a slow but considerable decrease in the light scattering reading was observed over a period of many hours (table 3d). Once a stable equilibrium value was reached after this fall, further addition of cholate brought about a decrease in the scattering, the equilibrium values all lying on a second smooth curve B, quite distinct from curve a. In fact the behaviour of these solutions is now identical with that described in section I, for solutions in presence of NaCl, *i.e.*, for a constant lecithin concentration, the addition of increasing quantities of cholate gave progressively lower equilibrium light scattering values.

The threshold value may be explained as follows. In preparations 1–5 of curve 'A' most of the cholate is engaged in the mixed micelles to disperse the lecithin. A fall in the light scattering reading sets in only when the amount of added cholate is of the order of the CMC (12 mM). The threshold value would then correspond more or less to the saturation of the inter-micellar solution with molecular cholate, and at the same time to a "saturation" of the mixed micelles present. With the addition of further cholate, the mixed micelles will be in equilibrium with a concentration of free cholate $>$ CMC, and the curve 'B' is obtained, which resembles the curves obtained in the presence of NaCl, (fig. 2A) the mixed micelles in that case also being in equilibrium with a concentration of free cholate $>$ CMC.

Below the threshold value, *i.e.*, when the concentration of cholate in the inter-micellar solution is $<$ CMC, the size of the "unsaturated" mixed micelles may not be diminished on addition of small amounts of cholate.

TABLE 4
Evolution of light scattering with time, effect of addition of Cholate crystals to an "unsaturated" mixed micellar system

Time (min)	10	160	220	280	1220	1520	2500
a) Sol. 1 (Lec. 0.01 M, Chol. 0.008 M)	2540	2700		2720	2920		
b) Sol. 1 + 0.004 M Cholate	3200	3250	3180	3220	3300		
c) Sol. 1 + 0.008 M Cholate	3740	3700		3680	3670		
d) Sol. 1 + 0.014 M Cholate	3540	2830	2700	2330	2060	2050	1880

b) *The action of simple electrolyte*

These aqueous solutions illustrate strikingly the difference between solutions in which the micelles are in equilibrium with a free cholate concentration either above or below the CMC. The behaviour in the course of time of a given solution (lec. 0.005 M, cholate 0.008 M) on the addition of different

NaCl solutions to the dry mixture, was followed by light scattering measurements (fig. 9).

When only low NaCl concentrations were added (0.02; 0.05; 0.07 M), coagulation took place, this being the more rapid as the concentration of the salt was greater. The rate of increase of scattered light per minute, with respect to a basic unit, has been calculated as a function of the salt concentration and a linear plot obtained in accordance with classical coagulation theory (inset fig. 9).

On the contrary, when a salt concentration of 0.2 M was added to the dry Lec-Cholate mixture, a steady, low light scattering value appeared to be attained almost instantaneously. With higher salt concentrations progres-

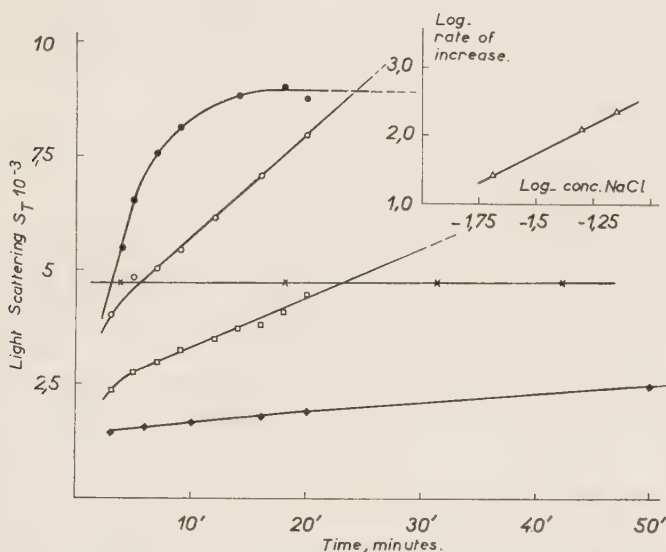


Fig. 9. Evolution of light scattering with time of a lecithin-cholate solution (Lec 5 mM, Cholate 8 mM) in the presence of different NaCl concentrations. NaCl: $\bullet\bullet$ 20 mM, $\square\square$ 50 mM, $\circ\circ\circ$ 70 mM, $\bullet\bullet\bullet$ 100 mM, $\times\times\times$ 200 mM.

Inset: Plot of Log (rate of increase of scattering light) vs Log (concentration NaCl).

sively lower, stable light scattering values were rapidly obtained, as illustrated previously in Fig. 2B. For an intermediate NaCl concentration, 0.1 M, there was an initial evolution of the solution, and then a steady value was reached.

The action of NaCl is twofold. By reducing electrostatic interactions it favours coagulation of "unsaturated" mixed micelles. At the same time the CMC of cholate is reduced, and if it falls below the value of the free cholate concentration present in the system, stable, saturated mixed micelles will be formed. In other words, if the quantity of NaCl added to a lecithin-cholate

solution is insufficient to bring the CMC of cholate below the value of the free cholate concentration, the system will coagulate.

V. Discussion

A. *Thermodynamic equilibrium of the system*

Although systems of mixed micelles have received much attention, relatively little is known of the variations in CMC and of the micellar weight with the proportions of additive present in the mixture. Previous investigations have been concerned with the maximum solubilisation of additive in the form of mixed micelles, and have generally ignored the variation in size of the dispersion below this solubilisation limit. It is the thermodynamic equilibrium governing the system below the saturation of the mixed micelles with lecithin which interests us particularly, given the enormous variation in mixed-micellar size observed (150000→10000).

Even for the case of a single ionic detergent the theoretical analysis of the variation of thermodynamic activity with concentration is highly complex, and we will only attempt a qualitative description of the present mixed system. Hoeve and Benson have proposed that the single-ion concentration increases above the CMC with increasing total concentration, and that this increase is probably accompanied by a shift to a larger micellar size¹¹). In the case of a classical detergent these variations may be small, but for small micelles, as those formed by cholate, any increase in the aggregation number is bound to correspond to an appreciable variation in activity and in the physical properties. The particular case of cholate has been examined in detail by Ekwall, who has shown that above the CMC the equilibrium monomer $\rightleftharpoons$ micelle varies over a wide concentration range, giving a noticeable increase in the cholate activity and in the solubilisation properties¹²).

Qualitatively the present system may be simply understood. These must be equilibrium between that part of the cholate engaged in the mixed micelles and that part which is dissolved in the micellar liquid, and the thermodynamic activity of the cholate must be the same in the two media. The size of the mixed micelles is the smaller the smaller the molecular ratio $N_L N_C$ in these micelles. At the same time the concentration of intermicellar cholate increases as the size of the mixed micelles decreases. Thus the degree of dispersion of the lecithin by the cholate is related to the activity of free cholate in the micellar solution. The addition of cholate to a given lecithin-cholate solution sets up a new cholate-monomer $\rightleftharpoons$ cholate-micelle equilibrium, with an increasing activity of the intermicellar solution. This provokes a change in the cholate activity in the mixed micelles, and smaller mixed micelles are formed corresponding to an increased cholate activity.

The minimum solubilisation of lecithin in cholate would correspond to the mixed micelles formed when lecithin is just dispersed by cholate (2L:1 chol), and we have seen in the presence of salt that this occurs more or less at a free cholate concentration close to the CMC of cholate. The free cholate concentration in equilibrium with the mixed micelles was shown to increase slowly with decreasing micellar size. Only in the presence of quite small mixed micelles does it increase more rapidly (fig. 7).

The importance of the inter-micellar solutions being saturated in molecular cholate was confirmed by experiments carried out to disperse dry lecithin by the addition of solutions of sodium cholate at different concentrations. A clarification of these mixtures was only obtained when sufficient cholate was present, in excess of the minimum needed in the mixed micelles to disperse the lecithin, to provide a free cholate concentration of at least the CMC (table 5). Similar results were observed with deoxycholate, for which the

TABLE 5
Solubilisation of dry Lecithin by Na Cholate solutions

Without NaCl(1)			NaCl 0.10 M constant (2)			Na Cholate 0.008 M constant (3)	
Cholate	Observation		Cholate	Observation		NaCl	Observation
0.010 M	O	3 weeks	0.006 M	Op	2 weeks	0.07 M	Op 10 days
0.015 M	Op	10 days	0.010 M	Cl	1 day	0.10 M	Cl 9 days
0.020 M	Cl	3 days	0.015 M	Cl	2 hours	0.20 M	Cl 4 days
0.030 M	Cl	15 min	0.020 M	Cl	30 min	0.25 M	Cl 36 hr

O=Opaque. Op=Opalescent. Cl=Clear.

Final Lecithin concentration 5 mM. Minimum Cholate in micelles 2.5 mM.

(1) CMC cholate 12 mM. Min. Chol.=CMC+2.5 mM=14.5 mM.

(2) CMC cholate 5 mM Min. Chol.=5+2.5 mM=7.5 mM.

(3) CMC variable. Min NaCl for solubilisation 0.1 M (CMC cholate 5-6 mM).

CMC in water is of the order 5 mM. The clarification is much more rapid when the amount of cholate present is well in excess of the CMC. The effect of NaCl may also be remarked. This lowers the CMC of cholate and permits penetration and dispersion of the lecithin by cholate at lower cholate concentrations.

Thus in principle it seems clear that lecithin may not be dispersed in the form of mixed micelles in the presence of an intermicellar solution which has a cholate concentration < CMC.

The existence of clear dispersions of lecithin in solutions where the cholate concentration in equilibrium with the mixed micelles is lower than its CMC

(see curve 'a', fig. 9) corresponds to a metastable state. The formation of such solutions may be simply explained in the following way. If to a concentrated lecithin–cholate solution with a free cholate concentration $> \text{CMC}$ a considerable amount of water is added, the free cholate concentration may fall below the CMC. The activity of the free cholate falls momentarily below that of the cholate in the mixed micelles, and some cholate will pass from the mixed micelles into the intermicellar solution. The mixed micelles are deficient in cholate, and hence their solubility will be diminished. The aggregation of these mixed micelles is thus favoured, and some form of aggregation may account for the very steep rise of the curve 'a', fig. 8. The existence of these stable "unsaturated" systems, whose formation is possible only by dilution of more concentrated solutions having a free cholate concentration $> \text{CMC}$, would seem to correspond to a metastable state. The addition of salt to these systems immediately provokes coagulation, as described previously.

B. *A model for the lecithin–cholate mixed micelles*

It is interesting to compare the micellar sizes estimated by light scattering with those of a model micelle, for equivalent L/C ratios. This model consists, as we have seen, in a bimolecular disc of lecithin, the outside lecithin chains being covered by cholate molecules, presenting the hydrophobic face of their steroid nucleus to the lecithin chains, and the hydrophilic face to the water. Thus "saturation" by cholate may consist in having a complete ring of cholate molecules at the surface of the lecithin-chains. In such a model both the top surfaces of the micelle, with the lecithin polar groups, and the sides, are hydrophilic (fig. 1). Some MWt values have been reported⁵), more detailed calculations are given below:

For a given $N_L N_C$ ratio, one may calculate the diameter of the model for which the lecithin core is completely covered by cholate, using the following dimensions of the molecules.

Area occupied by lecithin double chain, $A_L = 70\text{--}75 \text{ \AA}^2$ (maximum dimension of lecithin in the lamellar phase $70 \text{ \AA}^{213}$).

End-on area of cholate molecule, $A_C = 44 \text{ \AA}^{14}$).

The end-on view of a cholate molecule, as seen from a molecular model, is roughly in the form of an ellipse, of axes $2b = 7 \text{ \AA}$ and $2a = 8 \text{ \AA}$ ($\pi ab = \text{ \AA}^2 44$). The hydroxyl groups are in the side of the longer axis ($2a$), and this axis is thus oriented in the sense of the perimeter of the model.

Two equations may be constructed.

(i) Area occupied by N_L lecithin molecules in a lecithin core of diameter d_L

$$\pi/4 d_L^2 = N_L \times A_L \quad (6)$$

(ii) The number of cholate molecules N_C which can be spaced around the lecithin core at intervals of $8 \text{ \AA}$ ($2a$), taking a mean diameter of $(d_L + 2b) \text{ \AA}$

$$\pi(d_L + 2b) = N_C \times 2a. \quad (7)$$

Combining the two we obtain

$$d_L^2 = \left(\frac{d_L + 2b}{2a} \right) 4A_L \cdot \frac{N_L}{N_C}. \quad (8)$$

The micellar composition and weight have been calculated for various N_L/N_C ratios, taking MWts of 770 and 450 (with 1 molecule of water) for the lecithin and the cholate respectively, as in the light scattering calculations.

The MWt values, considering slightly different molecular models, are reported in table 5. The values of model are recorded as the broken curve in fig. 7. In the same figure MWt values determined by the method of analytical

TABLE 6
MW Values of Model Lecithin-Cholate mixed micelles

Molecular ration N_L/N_C in micelles	0.2	0.5	0.667	1.0	1.5	2.0
MW Model A	6.5	18.0	26.0	44.0	78.0	128.0
Model B	8.0	20.5	29.0	49.0	86.5	140.0
Model C	9.5	23.5	55.0	55.0	100.0	156.0
Molecular dimensions	Lecithin $\text{\AA}^2$		Cholate $\text{\AA}^2$ (πab)			
Model A	70		44 $\text{\AA}^2$ ($2a=8 \text{ \AA}$, $2b=7 \text{ \AA}$)			
Model B	75		44 $\text{\AA}^2$ ($2a=8 \text{ \AA}$, $2b=7 \text{ \AA}$)			
Model C	75		44 $\text{\AA}^2$ ($2a=7.5 \text{ \AA}$, $2b=7.5 \text{ \AA}$)			

centrifugation are recorded¹⁵). The values estimated from the light scattering data and the results of the centrifugation, are seen to be in good agreement with the model values, except for very high proportions of lecithin, where in fact the experimental measurements were the most difficult.

While there is no direct experiment evidence available in support such a model, it respects the normal condition of lecithin in the form of a bimolecular layer, at the same time making maximum usage of all the polar groups present. In the case of long chain alcohol solubilised by bile salts, Ekwall has also concluded that the alcohol molecules are ordered along the axes of the bile salt molecules¹²). The MWts as determined by light scattering on "saturated" solutions agree well with the model and the variation in micellar size as the Lec/Cholate ratio decreases is seen to be satisfactorily explained in terms of a layer of cholate molecules completely covering the outside surface of the lecithin core.

The maximum size of the micelles according to the proposed model is of the order 150×10^3 , and this size decreases to around 6000.

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